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Bioactive angiotensin peptides: focus on angiotensin IV

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Introduction

The renin-angiotensin system (RAS) was initially identified as a circulating humoral system with the effector peptide, angiotensin II (Ang II), generated by an enzymatic cascade. Angiotensinogen, which is synthesised in the liver, is cleaved by renin, a product of the juxtaglomerular cells of the kidney, to form angiotensin I (Ang I), which in turn is cleaved by angiotensin-converting enzyme (ACE) to form Ang II. ACE is membrane-bound and predominates on the endothelial cells of all vascular beds. Apart from the production of Ang II in plasma, Ang II, renin and ACE have all been described in tissues such as the brain, kidney, adrenal, vasculature, heart and ovaries. This suggests a separate and distinct RAS in these tissues and implies endocrine, paracrine and autocrine roles for Ang II.¹ A variety of other angiotensin peptides are also produced, although their activities have only recently received attention.

Angiotensin metabolism

Ang II regulates blood pressure (BP), fluid volume homeostasis and pituitary hormone release via AT₁- and AT₂-receptors located in the kidney, adrenal gland, and the cardiovascular and nervous systems.²⁻⁴ Ang II is rapidly metabolised in the circulation⁵ and in the cerebral ventricles.⁶ The aminopeptidases A and N sequentially remove amino acids from the N-terminus of Ang II to form the fragments Ang II (2-8), also known as Ang III, and Ang III (3-8), also known as Ang IV⁷ (Figure 1). Ang II (1-7) is another metabolite of Ang II,⁸ which may also be cleaved directly from Ang I⁸⁻¹⁰ by neutral endopeptidase 24.11 and prolyl endopeptidase¹¹ (Figure 1). Of these metabolites, Ang III binds with the highest affinity to AT₁-receptors (10-fold lower affinity than Ang II) while both Ang IV and Ang II (1-7) bind weakly (100- to 1000-fold lower affinity than Ang II).¹²

Ang III

Ang III exhibits most of the classical actions of Ang II, such as stimulation of aldosterone secretion, vasoconstriction and dipsogenic activity.¹³⁻¹⁵ In addition, recent findings suggest that Ang III may be an important agonist, if not the final mediator of Ang II actions in the central nervous system.^{7,16-18} This was clearly shown in studies by Zini *et al.*⁷ and Reaux *et al.*,¹⁹ in which the use of specific inhibitors of aminopeptidase A, required for the conversion of Ang II to Ang III, blocked Ang II-mediated vasopressin secretion and pressor

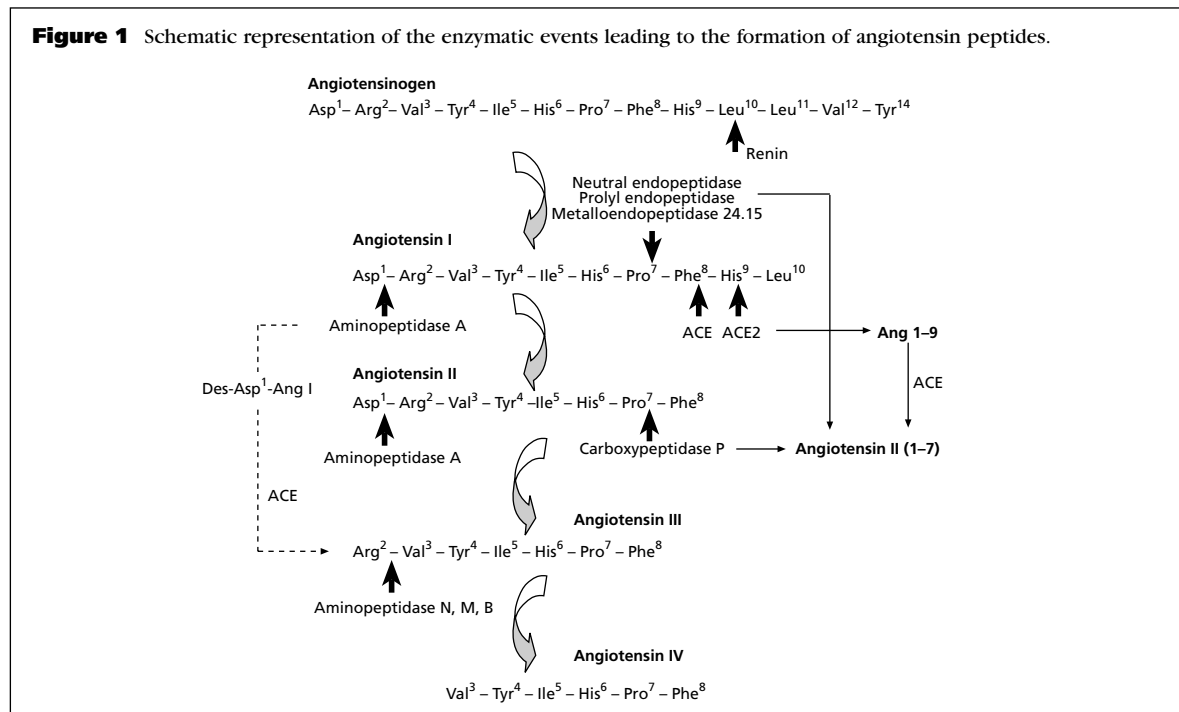
effects in rats. The ability of Ang III to mediate vasopressin release was confirmed by the ability of aminopeptidase A inhibitors to block Ang II-mediated increases in the firing rate of vasopressinergic neurones in the supraoptic nucleus.²⁰ Furthermore, preventing the breakdown of Ang III also resulted in large increases in BP, suggesting that Ang III may serve as the main effector hormone in the brain.²¹⁻²³ Ang III also up-regulates expression of TGF- β ₁ and fibronectin in renal interstitial fibroblasts²⁴ and increases monocyte chemo-attractant protein-1 (MCP-1) and transcription factors, nuclear factor κ B (NF- κ B) and activating protein-1 (AP-1).²⁵ These findings suggest a novel role for this heptapeptide in the pathogenesis of renal damage and disease.

In contrast to the actions associated with Ang III, initial work involving other Ang II metabolites, such as Ang II (1-7) and Ang IV resulted in the belief that these peptides were functionally inactive.^{13,14,26} However, it is now clear that both Ang II (1-7) and Ang IV are capable of eliciting a number of biological actions.

Ang II (1-7)

Ang II (1-7) can be formed by the enzymatic processing of Ang I or Ang II. Ang II (1-7) is thought to mediate a number of its actions by binding to a non-AT₁- and AT₂-receptor site that has yet to be cloned and characterised. This is based on pharmacological and physiological evidence suggesting that Ang II (1-7) is capable of mediating differential and opposing actions to Ang II, such as eliciting vasorelaxation in porcine,²⁷ canine²⁸ and feline²⁹ vessels, possibly via nitric oxide (NO) and/or prostaglandins.³⁰⁻³⁴ Ang II (1-7) has also been shown to potentiate bradykinin-induced vasodilatation in rats,^{35,36} as well as in canine²⁸ and porcine³⁷ coronary arteries. These actions are possibly mediated by a number of mechanisms, involving B₂ receptors, the novel Ang II (1-7) binding site,³⁵ regulation of ACE activity^{38,39} and/or NO and prostaglandin production.

Although Ang (1-7) does not stimulate aldosterone secretion or dipsogenesis, induce salt appetite or inhibit the baroreflex, it has been shown to inhibit angiogenesis⁴⁰ and proliferation of vascular smooth muscle cells,^{41,42} through a non-AT₁- and AT₂-receptor mechanism, opposing the angiogenic and proliferative actions of Ang II. The signalling mechanisms involved in the actions of Ang II (1-7) are also different to those of Ang II.^{30,34,43-45}

Figure 1 Schematic representation of the enzymatic events leading to the formation of angiotensin peptides.

The ability of Ang II (1-7) to mediate and oppose Ang II actions has been recently reviewed.⁴⁶⁻⁴⁹

Angiotensin 1-9 and ACE2

Recently, a novel ACE-related enzyme (ACE2) has also been discovered, that catalyses carboxypeptidase cleavage of Ang I to generate Ang (1-9), which subsequently serves as a substrate for the generation of Ang II (1-7). ACE2 is found on the endothelium of coronary and intrarenal vessels and renal tubular epithelium, raising the possibility that the formation of Ang II (1-7) may be important in regulating cardiac and renal function.^{50,51}

Angiotensin IV

Angiotensin IV (Ang IV), originally considered to be biologically inactive, has attracted recent attention because of its ability to elicit many biological actions. Ang IV binds with high affinity to a pharmacologically-distinct binding site, designated the AT₄ receptor.^{52,53} This binding site shows highest selectivity for Ang IV, with approximately 10-fold lower affinity for Ang III. Affinities for Ang II and [Sar¹-Ile⁸] Ang II are markedly lower, while losartan, PD 123177 and CGP 42112A show no activity.⁵⁴⁻⁵⁸ The AT₄-receptor has a distinct distribution pattern in the brain, being found in regions associated with cognitive, sensory and motor function in the guinea pig,⁵⁵ sheep⁵⁹ and monkey.⁶⁰ We recently localised the AT₄ receptor in human forebrain, midbrain, and pons, using an iodinated Ang IV analogue, Norleucine¹-Ang IV (Nle¹-Ang IV), a more stable analogue of Ang IV with higher affinity for the AT₄-receptor. The distribution of the AT₄-receptor in the human brain was similar to that reported in other species.⁶¹ The AT₄-receptor is also found in abundance in peripheral tissues, such as the heart, adrenal cortex, kidney, vascular smooth muscle cells and numerous other tissues.⁶²

Central actions of angiotensin IV

Initial interest in Ang IV was sparked by its ability to increase memory recall and learning in passive^{63,65} and conditioned avoidance response studies.⁶³ Treatment with Ang IV has been shown to improve memory in species as distant as crabs.⁶⁶ Infusion of Nle¹-Ang IV facilitated learning in the Morris water maze task in rats.⁶⁷ Furthermore, injection of Nle¹-Ang IV attenuated the deficit in spatial learning induced by a bilateral perforant pathway lesion in rats,⁶⁷ suggesting a trophic role for this peptide. The cognitive effects of Ang IV were thought to be mediated via the hippocampus, since intracerebroventricular administration of Ang IV induced *c-fos* expression in hippocampal pyramidal cells.⁶⁸ Ang IV and related analogues also potentiate potassium-evoked release of acetylcholine from rat hippocampal slices.⁶⁹ Recent studies demonstrate the ability of Ang IV and Nle¹-Ang IV to enhance long-term potentiation in the CA1 region of the rat hippocampus *in vitro*⁷⁰ and dentate gyrus *in vivo*,⁷¹ supporting a role for this peptide in memory processes.

Vascular actions of angiotensin IV

Several studies have focused on the ability of Ang IV to dilate vessels. In the rabbit, Ang IV caused vasodilatation of pial arterioles, but only if given subsequent to L-arginine administration, suggesting the involvement of NO.⁷² In support of this observation, internal carotid or renal infusion of Ang IV increased cerebral and renal cortical blood flows, respectively, actions which were blocked by pre-treatment with L-NAME.^{73,74} Ang IV caused an endothelium-dependent relaxation in porcine pulmonary arteries, in association with increased endothelial NO synthase (eNOS) activity and cGMP content in porcine pulmonary endothelial cells.⁷⁵ At high doses, Ang IV has been shown to mediate vasoconstriction via the AT₁-receptor.⁷⁶⁻⁸⁰

Cellular actions of angiotensin IV

To date, the most convincing data on the actions of Ang IV, in addition to cognition, have been on its mitogenic and trophic properties in a diverse range of cell types. Ang IV inhibits neurite outgrowth from cultured embryonic day 11 (E11) chicken sympathetic neurones⁸¹ and may therefore have a role in neuronal development. We recently demonstrated that Ang IV stimulates DNA synthesis by up to 80% in a human neuroblastoma cell line, SK-N-MC cells,⁸² suggesting a mitogenic role of Ang IV in neural cells. In a similar study using a rodent neuronal/glia hybrid cell line (NG108 cells), we found that an alternate ligand, LVV-haemorphin 7 (see below) was mitogenic, whilst Ang IV acted as a partial antagonist.⁸³ This demonstrates diversity in the actions of Ang IV in a species- and cell type-dependent manner. In addition to these trophic effects, Ang IV restores astrocyte adhesion, growth and morphology and prolongs the survival⁸⁴ and inhibits apoptosis of hippocampal cells⁸⁵ in angiotensinogen knock-out mice (AgKO).⁸⁶

Tropic actions of Ang IV have also been reported in rat anterior pituitary cells⁸⁷ and rabbit fibroblasts.⁸⁸ Furthermore, the peptide synergistically enhances basic fibroblast growth factor-induced DNA synthesis in cultured bovine endothelial cells.⁸⁸ More recently, intraperitoneal injection of Ang IV increased *in vivo* cell proliferation of rat endometrial epithelial cells labelled with bromodeoxyuridine (BrDU).⁸⁹ Ang IV also prevented developmental stage-specific renal papillary atrophy in AgKO mice, suggesting a role of the peptide in nephrogenesis.⁸⁶ In line with the trophic effects of Ang IV, we have observed an increase in AT₄-receptor binding by up to 150% in carotid arteries following balloon injury, suggesting a possible role of the AT₄-receptor in remodeling of the vascular wall following damage.⁹⁰

Signalling mechanisms of angiotensin IV

The signalling mechanisms associated with activation of the AT₄-receptor are still unclear. Attempts to clone the AT₄-receptor over the past several years have been unsuccessful and its exact identity has yet to be determined. The AT₄-receptor does not appear to be a G-protein linked seven transmembrane receptor like the AT₁- and AT₂-receptor subtypes. Cross-linking studies suggest that the AT₄-receptor in bovine tissue is a 160–180 kDa glycoprotein. Under non-reducing conditions, a second 225–250 kDa protein band is present, suggesting interactions of the receptor with smaller proteins that may be critical in signalling and function.^{91–93} Comparison of the size, and endopeptidase C digestion profile of the AT₄-receptor in selected bovine tissues suggested evidence for AT₄-receptor isoforms between peripheral and central tissue.⁹² However, in the human neuronal cell line, SK-N-MC cells, we demonstrated the size and glycosylation of the receptor to be consistent with those previously described in bovine tissue.⁸² GTP γ S binding, in a number of cell types,^{56,57,82} does not modify Ang IV binding to the AT₄-receptor,

further suggesting that this receptor is not associated with G-proteins.

Although the initial signalling events of the AT₄-receptor are unknown, centrally-administered Ang IV was reported to induce *c-fos* expression in regions of the rat brain involved with cognitive function.⁶⁸ By contrast, Nle¹-Ang IV decreased *c-fos* and *egr-1* mRNA in the mechanically-loaded, isolated rabbit heart.⁹⁴ In SK-N-MC cells (human neuroblastoma cells) stably transfected with a *c-fos* reporter gene construct, we were unable to show activation of *c-fos* following Ang IV treatment (unpublished observation). Although Ang IV has also been reported to increase the expression of plasminogen activator inhibitor-1 (PAI-1) mRNA in bovine aortic endothelial cells (five-fold)⁹⁵ and in human proximal tubular epithelial cells (three-fold),⁹⁶ this effect may be mediated by AT₁-receptors. Similarly, in human adipose tissue, Ang IV-mediated PAI-1 expression is blocked by candesartan.^{97,98} Furthermore, Ang IV stimulation of PAI-1 expression could not be demonstrated in rat aortic endothelial cells⁹⁹ and rat aortic smooth muscle cells.¹⁰⁰

Until recently, sparse evidence existed regarding the ability of the AT₄-receptor to regulate intracellular calcium concentrations. In NG108-15 cells¹⁰¹ and embryonic chick cardiac myocytes,¹⁰² Ang IV does not alter intracellular Ca²⁺ levels [Ca²⁺]_i. In rat vascular smooth muscle cells, Ang IV produced a small, but sustained increase in [Ca²⁺]_i via extracellular influx, and an increase in inositol phosphates,¹⁰³ while in the opossum kidney cell line, Ang IV stimulated a transient increase in [Ca²⁺]_i (via voltage-sensitive channels), independent of inositol-phosphates.¹⁰⁴ Since then, a number of studies have reported the ability of Ang IV to stimulate [Ca²⁺]_i in cells of pulmonary, cardiac and renal origin; Ang IV dose-dependently increased [Ca²⁺]_i in rat mesangial cells through influx of calcium from the extracellular media or internal stores.¹⁰⁵ In human proximal tubule epithelial cells, both Ang IV (10 nM) and its proposed antagonist, divalinal-Ang IV (1 μ M), also increased [Ca²⁺]_i. Interestingly, in the same study both peptides resulted in a rise in intracellular Na⁺, and increased levels of phosphorylated Erk-2, while Ang IV alone stimulated kinase p38 activity. In porcine proximal epithelial cells, Ang IV (1 μ M) failed to affect cytosolic calcium concentrations, cAMP or cGMP production, but did cause dose-dependent tyrosine phosphorylation of a focal adhesion protein (p125-FAK).¹⁰⁶ Both Ang IV and divalinal-Ang IV also increased [Ca²⁺]_i in bovine kidney epithelial cells (MDBK).¹⁰⁷

In association with the ability of Ang IV to increase intracellular calcium levels, Ang IV was also shown to increase NO synthase (NOS) activity rather than protein expression in porcine aortic endothelial cells.^{75,108} This effect was abolished in the presence of intracellular calcium chelators, suggesting that Ang IV mediates regulation of NOS activity via calcium. In association with an increase in NOS activity, cGMP production was also increased in porcine aortic endothelial cells following Ang IV treatment, and this was abolished in the presence of L-NAME. In the same study, Ang IV

modulated expression of calreticulin via calcium.

In summary, Ang IV, particularly in porcine aortic endothelial cells, appears to increase intracellular calcium, which in turn increases NOS activity with subsequent production of NO and cGMP. In contrast, Ang IV failed to alter basal guanylate cyclase activity in opossum kidney cells,¹⁰⁴ and porcine proximal epithelial cells,¹⁰⁶ suggesting tissue-specific actions of the AT₄-receptor system.¹⁰⁴

Endogenous ligands of the AT₄-receptor

Whether Ang IV is the true ligand for the AT₄-receptor in the brain is doubtful, since the localisation of the components of the RAS in the brain do not correspond with its distribution. We identified an alternative ligand for the AT₄-receptor, a decapeptide identical to an internal sequence of the sheep (amino acids 30–39) and human (amino acids 32–41) β-globin. The globin fragment, termed LVV-hemorphin-7 (LVV-H7), was isolated from sheep brain, using an AT₄-radioreceptor binding assay and a multistep protein purification procedure.¹⁰⁹ This abundant peptide (approximately 2 nmol of peptide per gram of tissue) exhibits very high affinity for the AT₄-receptor. Both Ang IV and LVV-H7 display identical binding patterns in the sheep brain.¹⁰⁹ LVV-H7 has also been previously isolated from the brain of other species,^{110–113} reflecting the abundance of the peptide in the central nervous system. Furthermore, the enzymes pepsin,¹¹⁴ trypsin¹¹² and a high molecular weight aspartic proteinase present in bovine brain¹¹¹ are all capable of processing β-globin to LVV-H7.

Apart from inhibiting opioid activity,¹¹⁴ our studies indicate that LVV-H7 also inhibits neurite outgrowth from E11 chicken sympathetic neurons and stimulates DNA synthesis in both rodent and human neural cell lines such as NG108 cells⁸⁵ and SK-N-MC cells.⁸² Like Ang IV, LVV-H7 dose-dependently enhances depolarisation-evoked release of acetylcholine from rat hippocampal slices.⁶⁹ Although the central actions of LVV-H7 appear to be consistent with Ang IV, the peripheral actions are not. For instance, LVV-H7 showed no effects on [Ca²⁺]_i in rat mesangial cells¹⁰⁵ and had no significant effect on renal blood flow, mean arterial pressure or heart rate in rats infused with 0, 100 and 1000 pmol/min LVV-H7.¹¹⁵

Conclusion

For many years, most attention has revolved around Ang II and its undoubtedly important roles in the regulation of BP and fluid volume homeostasis and cardiovascular structure and function. However, recent work suggests that Ang II metabolites may also be of physiological importance. It is now clear that peptides such as Ang III and Ang II (1–7) may be important in mediating some of the actions that have formerly been attributed to Ang II. Recent studies clearly suggest that Ang III may be an agonist in the brain, mediating at least some of the actions of Ang II. Ang (1–7) is somewhat more complex, capable of mediating, or opposing, Ang II actions through a novel non-AT₁/AT₂-receptor site, and by its interaction with other receptor

systems. It is now evident that Ang IV binds to a specific binding site, designated the AT₄-receptor, that displays low affinity for Ang II receptor agonists and is differentially distributed in the central nervous system, with actions independent of the other active angiotensin fragments. An alternate peptide ligand of the AT₄-receptor, LVV-H7, in the brain, suggests the AT₄-receptor may be part of a novel neuropeptide system involved in memory and neural development. Overall, the RAS is emerging as a system with multiple effector peptides with diverse functions.

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